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TB50: Intestinal Absorption of Amino Acids In Vitro with Special Reference to the Chicken: A Review of Recent Findings and Methodological Approaches in Distinguishing Transport Systems

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Intestinal Absorption of Amino Acids *In Vitro* With Special Reference to the Chicken: *A Review of Recent Findings and Methodological Approaches in Distinguishing Transport Systems*

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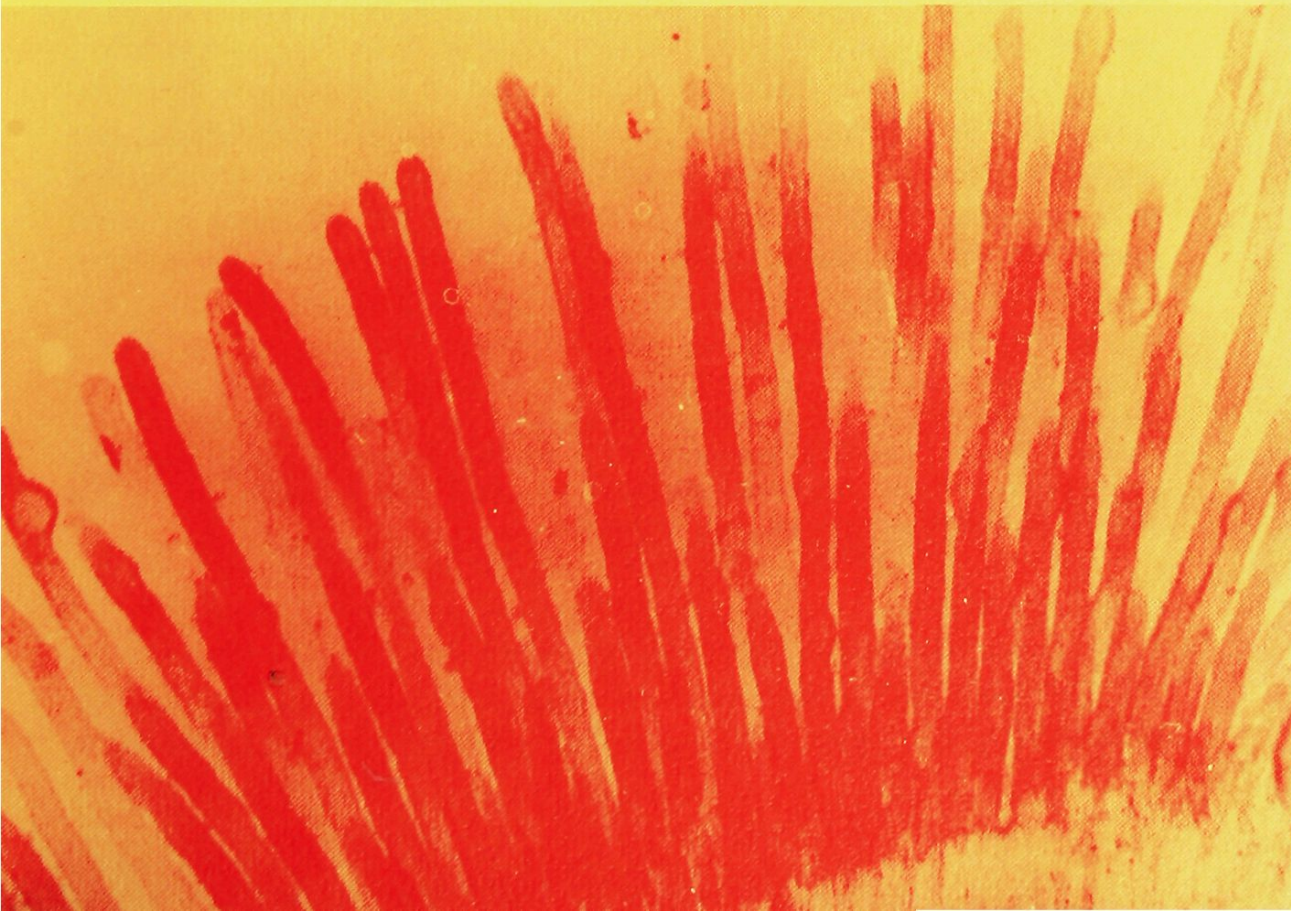

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Electron micrograph of membrane bounding the intestinal lumen of the chicken. Magnification is approximately 50,000 diameters. The finger-like projections contain numerous digestive enzymes and presumably are the site of the transport systems for nutrients.

INTESTINAL ABSORPTION OF AMINO ACIDS *IN VITRO* WITH SPECIAL REFERENCE TO THE CHICKEN:

A Review of Recent Findings And Methodologial Approaches in Distinguishing Transport Systems

Joseph Lerner

The potential importance of the work reported in this bulletin centers about the need for a complete description of the initial step in the metabolism of amino acids, namely, their interaction or recognition by the brush border membrane which lines the intestinal lumen. Hence, I have undertaken to review the current status of amino acid absorption from intestine and have placed particular emphasis upon the studies done here at Orono with chicken intestine. What emerges from the numerous studies is a complex picture of how amino acids interact in their competition for the various transport systems which allow their movement into the epithelium. To this point, this bulletin describes the characteristics of a number of such systems which specifically function in the regulation of amino acid absorption. Further, it is addressed to a consideration of the technical problems that the investigator confronts in describing the transport of a given substrate. In particular, this publication outlines the major criteria by which one defines a so-called transport system. As opposed to enzyme chemistry, the procedures found in this report are indirect; whereas, in enzymology a scientist may isolate in pure form a biological catalyst which we call an enzyme, no one to date has isolated and retained catalytic activity of a so-called transport system. Perhaps, such difficulties are to be expected when one removes from the membrane those components which have as their function the vectorial operation of transport. Despite inherent difficulties, much indirect information has been gained. Ultimately, an understanding of the function and control of the initial stages in the metabolism of amino acids in the intestine will provide a basis for correcting pathological states of such metabolism and for improving the efficiency of utilization of food materials.

TISSUE INCUBATION PROCEDURES

Chickens, 7 to 14 weeks of age, were fasted 24 hours prior to sacrifice and were then killed by cervical dislocation. A portion of small intestine approximately 3 cm long on either side of the yolk stalk (or at the point of consideration) was excised and immersed in previously

gassed (O_2:CO_2, 95:5% by vol) physiological saline enriched with 0.3% glucose. This solution was maintained at 37° The intestine was manually stripped of mesentery and fatty tissue and then cut into eight segments, each weighing about 100 mg. The latter were then cut lengthwise and allowed to contact towel paper to remove excess fluid. Even-numbered and odd-numbered segments of tissue were segregated and incubated at 37° with shaking in 25 ml erlenmeyer flasks containing gassed (O_2:CO_2, 95:5% by vol) 5 ml portions of Krebs-Henseleit buffer containing test C^{14}-labeled amino acid. We have found that highly reproducible results (15 sec minimum incubation time) could be obtained in short incubation periods if the sections of tissue were first placed in the constricted portion (mouth) of the flask and the rubber stopper inserted in the flask. The reaction was started by rapidly shaking down the sections of tissue into the incubation medium. The reaction was terminated by pouring the flask contents onto a funnel (maintained under suction) and simultaneously washing the tissue with Krebs-Henseleit buffer (sprayed from a wash bottle). The segments were then rapidly removed to towel paper and the excess buffer removed by rolling; they were then placed on a Kimwipe, which was folded, and the sandwiched tissue gently kneaded to remove residual fluids by capillary action.

For experiments where incubations were shorter than 15 sec, a modification of the procedure developed by Schultz and Curran (1969) was used.* Briefly, tissue was incubated for the desired time on a Hirsch funnel which was inserted into a vacuum flask. The reaction was monitored with a clock system that was coupled to a relay, which opened or closed a vacuum line communicating with the flask. The incubation was started by pouring the preheated (37°) and pregassed 5 ml portion of amino acid solution onto the filter, and was terminated when the relay allowed a strong vacuum to evacuate substrate solution from the funnel. The tissues were simultaneously irrigated with Krebs-Henseleit buffer. They were than dried of adherent fluids as mentioned above, weighed and ground in a Potter-Elvehjem tissue grinder in a portion of 2.5% trichloroacetic acid, using 5 ml of this solution per gram of tissue. The tissue extracts were centrifuged at 49,500 x g for 30 min and the labeled amino acid assayed by counting a 0.2 ml portion of the clarified extract in Bray's solution (1960). Uptake values were routinely expressed as micromoles accumulated per ml extract per time interval of incubation. Other details of methodology are to be found in previous publications (Lerner and Taylor, 1967; Lerner *et al.*, 1968; Lerner *et al.*, 1969; Nelson and Lerner, 1970; Herzberg *et al.*, 1971; LaBelle *et al.*, 1971). The kinetic theory used as a basis for many of these studies has been described in a recent review (Lerner, 1971).

* Contribution from the Ph.D. thesis of David S. Miller.

RECENT FINDINGS

Absorption of Neutral Amino Acids

We have shown that absorption of methionine from the chicken small intestine is inhibitable in the order of increasing amino acid side-chain length, and that if one instead uses glycine as substrate, that alanine is seen to be its best inhibitor, followed by the long-chain aliphatic amino acids (Lerner and Taylor, 1967; Nelson and Lerner, 1970). Similarly in the rat small intestine, methionine transfer has been found to be progressively inhibited with increasing chain length, while sarcosine is inhibited less by long-chain compounds, and more by glycine and alanine (Daniels *et al.*, 1969). Observations such as these have provided the basis for proposing the existence of two separate mechanisms for neutral amino acid transfer in rat intestine, a methionine system stereospecific for L-isomers of amino acids, and a nonstereospecific sarcosine system (Daniels *et al.*, 1967; Newey and Smyth, 1964), and in chicken intestine, a methionine system and a glycine system, both having preference for L-isomers (Nelson and Lerner, 1970). Likewise, a distinct betaine transport agency has been described for hamster intestine that is shared with sarcosine, proline and hydroxyproline (Hagihira *et al.*, 1962). According to Munck (1966), proline, alanine and leucine in rat intestine have affinities for both the long-chain neutral receptor and the imino-glycine (sarcosine) system. On the other hand, Newey and Smyth (1964) in their work with everted sacs of rat intestine found methionine, leucine, glycine and proline to be handled by the methionine system, while the absorptions of glycine and proline (but not methionine or leucine) were also effected by the sarcosine system. De La Noue *et al.*, (1969) found alanine to be handled preferentially by the methionine system in the rat, while β-alanine is transported by the sarcosine system. In contrast, the system transporting glycine in the chicken shows a marked requirement for a free α-amino group; hence, proline, hydroxyproline, β-alanine and sarcosine appear to be excluded (Nelson and Lerner, 1970). For the site absorbing glycine, the affinities of neutral amino acids are about one order of magnitude smaller than for the methionine site, while both sites have little or no affinity for either basic or acidic amino acids.

The interaction of long-chain neutral amino acids with proline is less intense than with alanine (Lerner and Burrill, 1971); furthermore, both sarcosine and β-alanine (which have no inhibitory effect on alanine uptake) act to impede the flux of proline. These results are preliminary indications that alanine is probably transported mainly via the methionine system (see also, Nelson and Lerner, 1970), and that proline is perhaps partitioned into the methionine system and another system, which in analogy to the sarcosine system in the rat, accepts sarcosine

and β-alanine. The uptake of β-alanine in our recent work is seen to be weakly inhibited by the major substrates of the methionine system, and the effects of aliphatic hydroxy amino acids (threonine, serine and hydroxyproline) on β-alanine are conspicuously weak, the best inhibitors being D- and L-proline, sarcosine, alanine and β-alanine. (Lerner and Burrill, 1971). When an excess of leucine is taken to block the transport of proline in the methionine system, the various inhibitors of proline absorption provide a glimpse of the specificity of a proline transport system unshared with leucine. Under these circumstances, only β-alanine, hydroxyproline, sarcosine, D-proline and the azetidine carboxylic acid stereoisomers inhibit proline above the effects caused by saturating levels of leucine. The reactivities of β-alanine and hydroxyproline are relatively weak when analyzed this way (P. Burrill and J. Lerner, in press). Noteworthy is the fact that glycine is not an inhibitor; thus, the transport of glycine and that of proline are apparently more separate with respect to the transport agencies as compared with findings in the rat (Daniels *et al.*, 1969).

The glycine system which we have characterized (Nelson and Lerner, 1970) cannot be inhibited by β-alanine, α-aminoisobutyric acid (AIB), sarcosine, or proline to any appreciable extent. AIB, on the other hand, does inhibit β-alanine transport. Our data (Lerner and Burrill, 1971) which show D- and L-proline to have the same effectiveness in inhibiting β-alanine uptake are reminiscent of the findings of Daniels *et al.* (1969) that the proline isomers reduce sarcosine entry into rat intestine equally. More recently, the order of inhibitions of proline in leucine-blocking experiments shows that, whereas D-proline is as potent as L-azetidine carboxylic acid (both being the most effective of the substances tested), D-azetidine carboxylic acid is less reactive than its enantiomer (P. Burrill and J. Lerner, in press).

While sarcosine has proven to be a useful substrate in delineating the so-called sarcosine system in rat small intestine (Daniels *et al.*, 1969), our data (Lerner and Burrill, 1971) show that its overlap in what appears to be a number of transport agencies is great, making it a poor choice for studying transport in a given agency. Again, this is an example of species-specific differences in transport receptor sites.

To review, we point to glycine as having transport properties apart from proline and other imino acids in chicken intestine, while in the rat, the various receptors, as shown by the work mentioned above, do not appear to distinguish between glycine and the imino acids. On the contrary, we hypothesize at present that chicken intestine possesses at least three neutral agencies, one for methionine and long-chain neutral amino acids, one for glycine, and one for β-alanine, proline and related imino acids. With respect to hamster intestine we quote from an abstract of

Tager *et al.* (1971): "Although uptake of methylAIB is inhibited by various α-amino acids, uptake of leucine or 2-aminonorbornane-2-carboxylic acid (BCH) is scarcely affected by the presence of 0.04 M methylAIB, a behavior we attribute to limitation of reactivity of the latter to the so-called iminoglycine system. Uptake of the levorotatory isomer, (—)b-BCH, is much more easily inhibited by various amino acids, especially those with large apolar sidechains, than is uptake of (+)b-BCH. For example the ratio of the uptake seen in the presence of glycine to the uptake in the presence of phenylalanine or methionine is 2 or 3 times as large for the levorotatory isomer. Since methylAIB has little effect in either case, this result points to the contribution of a third agency."

Further evidence in support of the contention that glycine transport may occur via a system independent from that serving the imino acids is seen in data obtained for rabbit renal tubules where glycine mediation occurs by at least three separate routes, one shared with alanine, a proline-shared system, and one unshared with either compound (Hillman *et al.*, 1968). In the pigeon, red cell glycine enters by a highly specific route which accepts only N-methyl and N-ethyl derivatives of glycine (Vidaver *et al.*, 1964). These observations illustrate that in context of biochemical comparisons between species, the glycine transport agency is discrete from imino acid accepting systems in certain cases but merged in others. Theoretically, one might be confronted with two separate agencies with overlapping specificities which give the appearance of one agency with broad specificity. Another complicating factor is that found by Peterson *et al.* (1970) in which glycine enters rabbit ileum by an absorption mechanism in common with other monoamino-monocarboxylic acids, while its uptake by a separate mechanism in common with proline has a capacity of only 5% of the major pathway. In our work, glycine shares transport with other neutral amino acids in a system that appears to have about 1/10 the tolerance for accepting such substrates as does the methionine agency (Nelson and Lerner, 1970).

Lastly, in our preliminary experiments with taurine absorption in chicken intestine (Lerner and Burrill, 1971), we noted its transport to be complex with respect to the various receptor sites. While it inhibits alanine transport to a small extent, the absence of effect upon glycine transport is conspicuous. Further, it has no effect upon proline entry, though it can be inhibited best by taurine, proline and glycine, and least by methionine, alanine and isoleucine. Its major route of uptake clearly is not the methionine system.

Absorption of Cationic Amino Acids

We have recently investigated the absorption of basic amino acids from the chicken small intestine (Herzberg *et al.*, 1971). These substances appear to compete for either a common pathway in transport

or in a cluster of functionally related sites (in analogy perhaps to the reactivity of isoenzymes) with an order of reactivity: homoarginine $\simeq$ arginine $\simeq$ lysine $\simeq$ ornithine $>$ diaminobutyric acid (DAB) $>$ histidine. The possibility of hypothesizing the cluster of sites concept arose out of considerations of our data which showed dissimilarities between K_m for given substrates and their respective K_i values measured in cross inhibition studies (Herzberg *et al.*, 1971). The basic amino acid pathway does not serve for the major component of histidine entry because the most reactive basic amino acids are only mild inhibitors of histidine. DAB, on the other hand, is strongly inhibited by lysine, indicating probable activity with that pathway. Both histidine and DAB are very poor inhibitors of either alanine or methionine absorption, facts which rule out their entry into the methionine system (Herzberg *et al.*, 1971). Chez *et al.* (1971) report for rabbit ileum that histidine influx is indeed complex with respect to its inhibitions by alanine and lysine, especially since the interaction in the presence of the latter substances is independent of pH (i.e., despite the fact that histidine changes form from being a cation to an α-zwitterion).

We have found that the neutral amino acids alanine, proline and leucine have the same V_{max} within experimental error; histidine and DAB also have this value. Moreover, the strongly basic amino acids have a common V_{max} (Lerner and Burrill, 1971; Herzberg *et al.*, 1971). We theorize that DAB and histidine may pass through the cell membrane as α-zwitterions. Nevertheless, the strong inhibition of DAB by lysine suggests that the binding reaction for DAB may involve its cationic form (Herzberg *et al.*, 1971). The results of Chez *et al.* (1971) show that the kinetics of histidine entry and the interactions with alanine or lysine are not affected by the charge distribution of histidine in the incubation medium; they offer two explanations for the observed results: The net charge on histidine does not affect the interaction between histidine and its receptor or the dependence of this process on Na^+. They state that charge alone cannot be the determining factor in selectivity. Alternatively, they consider that the ionic form in which histidine reacts with the transport system is not determined by the form of histidine in bulk solution, because the microclimate of pH in the zone adjacent to the brush border may have a pH of 5 or 6 and that this value is only slightly influenced by large changes in pH in the medium.

We have observed (Herzberg *et al.*, 1971) that the 5 min rates of uptake for methionine, glycine, leucine, proline, alanine and histidine found under anaerobic conditions, though reduced when compared with aerobic conditions, are significantly greater than the rates obtained for these substances when they are incubated in a Na^+ -depleted buffer. On the other hand, the strongly basic amino acids and DAB show rates

which do not differ between experimental conditions. From this one might speculate that the Na^+ gradient for transfer of cationic amino acids is tightly coupled to aerobic metabolism, while for the neutral amino acids, energy for the Na^+ pump derives not only from aerobic metabolism, but from energy produced anaerobically as well. It is our contention that the effects of oxygen depletion and Na^+ depletion are specific for these two classes of compounds (Herzberg *et al.*, 1971).

While many neutral amino acids have been shown to inhibit basic amino acid transport in various cellular and tissue systems, the reciprocal reaction has generally not been observed to occur (Herzberg *et al.*, 1971). Recently we found that homoarginine could not inhibit the transport of leucine in uptake experiments done at 5 sec incubation and 5 min (LaBelle *et al.*, 1971). However, in a study by Reiser and Christiansen (1970) some strongly basic amino acids were discovered to inhibit neutral amino acid transport by as much as 50% in rat when the inhibitor was present in excess (at 25 mM). With regard to this matter, Christensen *et al.* (1969b) have cautioned that at high basic amino acid concentration, sufficient α-zwitterion is present to effect an inhibition in the long-chain neutral amino acid transport system.

It appears, within experimental error, that the inhibition of the 5 min flux of arginine in the presence of leucine in chicken intestine is of the classical competitive type (Herzberg *et al.*, 1971). Similarly, Reiser and Christiansen (1969), working with rat intestine, showed that valine is a competitive inhibitor of lysine (5 min flux). The latter workers further qualified the nature of such effects by examining the 30 min accumulation of lysine in the presence of valine; under these circumstances, the inhibition reached a maximum at 75% (as opposed to complete inhibition effected by arginine). Hence, they concluded that valine was a partial competitive inhibitor. They also reported that lysine reduces the uptake of valine only 10-15% while in excess concentration (25-100 mM), and that such inhibition was not found to be competitive. They have concluded that neutral amino acids bind to a closely associated site on a single or polyfunctional carrier and bring about an allosteric type of modification of the basic amino acid transport site.

In our investigations (Herzberg *et al.*, 1971), we have shown the effect of arginine on the 5 min flux of leucine to be slight. Additionally, while the transport of leucine is considerably more Na^+ -dependent than that of lysine, it is of interest that in the absence of Na^+ from the incubation medium, the inhibition of arginine uptake as caused by leucine is significant; under the latter conditions, lysine has negligible effects. The aspect of the Na^+ - independence of the effect of leucine on arginine uptake is akin to the Na^+ independence of leucine stimulation of lysine influx across rabbit ileum preincubated in a leucine-containing medium

(Munck and Schultz, 1969a,b). In that study, the stimulatory effect was noted to be due to an increase in the unidirectional flux of lysine out of the epithelial cell, across the serosal and/or lateral cell membranes; these authors suggested that leucine acts in a noncompetitive manner on the carrier mechanism for lysine movement across the serosal boundary of the epithelium. Moreover, they found that preloading rabbit ileum with leucine enhances unidirectional influx of lysine; preloading with lysine has no effect on lysine influx, and preloading with leucine has no effect on leucine influx. Methionine to a lesser extent caused lysine stimulation though none was seen with isoleucine, valine, serine, aspartic acid or sarcosine. They state further that although similar transport properties are seen between leucine and a number of these compounds, the specificity of leucine with respect to stimulation may be a property of it unrelated to its transport. Scriver and Mohyuddin (1968) have described a similar interaction in rat kidney (in long-term incubation experiments) that occurs between proline and AIB; the effect of proline appears to be highly specific and unrelated to its transport. Reiser and Christiansen (1969) observed that both leucine and methionine, and to a lesser extent valine, cause stimulation of the 30 min uptake of lysine in rat small intestine; glutamate and valine produce about equal activation, but arginine inhibits lysine under these conditions (both lysine and the test amino acid were present in the incubation medium at 1 mM). Further, they found the stimulatory phenomenon to be abolished when the concentration of leucine was increased to 5 mM.

The inhibition of basic amino acids by long-chain neutral amino acids has been noted widely in both tissue and isolated cellular systems (see Herzberg *et al.*, 1971). The findings of Reiser and Christiansen (1969) and Larsen *et al.* (1964) for rat intestine show a graded response of neutral amino acids with the best inhibitors being the long-chain species, which are about as effective as the basics themselves, followed by valine and then glycine and proline; this order of action parallels the activities of the same compounds in their inhibition of the "neutral amino acid carrier" in the view of these workers. In the chicken intestine, Herzberg and coinvestigators (1971) have discovered that the long-chain and branched-chain neutral amino acids react strongly to modify cationic amino acid transport. Leucine affects arginine transport to approximately the same extent as do lysine and ornithine. Other substances of moderate to strong reactivity in the inhibition of either lysine or arginine include phenylalanine, norleucine, homocitrulline, valine and ethionine. Further, we have found that glycine, proline, β-alanine and AIB have little effect on lysine transport in chicken intestine. Low activities were also noted with serine and homoserine; threonine did not inhibit. In contrast with the study of Reiser and Christiansen (1969) in which

they found parallel effects of neutral amino acids when these substances are used to inhibit either basic or neutral transport in the rat intestine, we have seen contrasting patterns of inhibition when many neutral amino acids are used, in turn, to diminish the uptakes of methionine and lysine, respectively (Herzberg *et al.*, 1971). Thus, while phenylalanine and tryptophan, in our system, are among the most potent of the neutral amino acid inhibitors of lysine, they are not in the class of best inhibitors of methionine. α-Amino-n-butyric acid (ANB) and serine have either little or no effect on lysine absorption, and threonine, which moderately decreases methionine influx, instead stimulates lysine influx. The methionine site appears to tolerate interaction with aliphatic hydroxy species, whereas that side-chain configuration is poorly received in the lysine pathway. Additionally, we have reported in the work of Herzberg and coworkers (1971) that excess serine, when used to block the methionine site from reactivity with phenylalanine, cannot reverse the inhibition of lysine influx caused by phenylalanine. From this type of reasoning, we have concluded that the methionine site is probably not the point of attachment of bulky, neutral amino acids when they act to diminish lysine uptake.

Our current data suggest that little if any transport of leucine occurs in the basic amino acid pathway, as judged from the negligible modification of its entry seen in the presence of arginine (Herzberg *et al.*, 1971). We cannot preclude the possibility that leucine may act to inhibit arginine transport in a competitive fashion, yet not undergo transport via that system (Christensen *et al.*, 1969a).

Some recent developments in the area of basic amino acid transport are worthy of brief mention. Employing everted sacs of rat intestine, Reiser and Christiansen (1971) found that the transport of 1 mM lysine was stimulated by either 1 mM leucine, 1 mM methionine or 1 mM alanine in the medium; under these circumstances, the intracellular concentration of lysine did not increase as the concentration in the serosal solution increased. They also reported that the uptake of 1 mM lysine and 1 mM arginine by isolated epithelial cells is stimulated by 1 mM leucine, 1 mM methionine, 1 mM alanine or 1 mM phenylalanine; inhibited by 1 mM isoleucine or 1 mM tryptophan; and unaffected by 1 mM valine, 1 mM histidine or 1 mM glycine. Of considerable interest, the stimulation of lysine transport in the isolated cells is present after a 1 min incubation and optimum after 2 min. The inhibition of 1 mM leucine uptake by 1 mM lysine is also maximal after 2 min. Furthermore, they state that preincubation of cells in 1 mM lysine, 1 mM arginine, or 1 mM leucine followed by incubation of lysine results in an increased absorption of lysine relative to a Krebs-Tris buffer. The stimulation of 1 mM lysine uptake by leucine preloaded cells is not decreased by ex-

clusion of medium Na^+. These investigators have summed up these data in the following hypothesis: The stimulation of lysine transport in the presence of leucine in the cells takes place by function of two transport systems. One mediates an energy-dependent, Na^+ -dependent active accumulation of leucine and the other, a Na^+ -independent hetero-exchange between intracellular leucine and extracellular lysine which causes stimulation of lysine uptake at the expense of intracellular leucine.

This year we determined that there is no significant cross interaction between representative neutral and basic amino acids in 5 sec uptake experiments (LaBelle *et al.*, 1971). Thus, the apparent initial reactions of these classes take place in chicken intestinal epithelium by separate processes. Morever, the one-way reaction between leucine and arginine was found to be time-dependent which may indicate some degree of spacial separation between their site of interaction and the sites involved in the entries of these compounds into the epithelial cells, Thus, the previously observed interaction in which neutral amino acids diminish the uptakes of basic amino acids in 5 min experiments can now be attributed to a process that is separate from the apparent initial event.

Absorption of Acidic Amino Acids

The absorption of dicarboxylic acids from intestine has been an under-developed area of endeavor, primarily because early workers failed to observe a movement of aspartic acid or glutamic acid against their respective concentration gradients, and because of the finding that these substances are rapidly transaminated by the tissue (Wiseman, 1953; Matthews and Wiseman, 1953; Neame and Wiseman, 1957; Lin *et al.*, 1962; Spencer *et al.*, 1966; Neame and Wiseman, 1958; Peraino and Harper, 1962). Recently, Schultz and coinvestigators (1970) have succeeded in studying the transport of these substances in very short time periods (1 min uptakes), and have overcome the various complicating factors due to metabolism of these subtrates. Their report shows that acidic amino acids enter intestinal tissue of rabbit ileum via a carrier-mediated process wherein their entry rates are saturable, Na^+ -dependent, and subject to inhibition by aspartic or glutamic acid. These discoveries support previous observations which had hinted at the likelihood that intestine contains an acidic carrier mechanism. Thus, Gibson and Wiseman (1951) found both aspartic and glutamic acids to be absorbed at rates comparable to those of neutral amino acids and that the L-isomers were seen to enter faster than their D-antipodes.

In 1955, Wiseman observed glutamate to be ineffective in causing the inhibition of methionine transport from equimolar concentrations in hamster intestine; the data show stimulation of methionine transport instead. We found aspartate to be incapable of inhibiting methionine uptake in chicken small intestine (Lerner and Taylor, 1967); both aspar-

tate and glutamate are insignificant inhibitors of glycine transport in this tissue (Nelson and Lerner, 1970). Moreover, glutamate stimulates lysine transport in rat intestine (Reiser and Christiansen, 1969) and does not appreciably inhibit the flux of glycine in Ehrlich cells (Christensen *et al.*, 1952; Heinz *et al.*, 1965). Nevertheless, the entry of glutamate into Ehrlich cells is a saturable process which can be inhibited most effectively by long-chain neutral amino acids, but less by glycine, and insignificantly by other acidic amino acids (Heinz *et al.*, 1965). Additionally, Webber (1962) discovered neutral amino acids to be capable of inhibiting acidic amino acids in renal tubules in the order of increasing side-chain length in the series, glycine, alanine and methionine.

APPROACHES TO DISTINGUISHING BETWEEN SEPARATE TRANSPORT PATHWAYS

A rigorous test has been proposed by Ahmed and Scholefield (1962) which can be used to show that the uptake of two substances *A* and *B* occurs by attachment to a common membrane site. Three conditions must be fulfilled: (1) *A* must be a competitive inhibitor of the transport of *B*, and *B* must be a competitive inhibitor of the transport *A*. (2) The apparent Michaelis constant for the transport of *A* must equal its K_i when acting as an inhibitor of *B* transport, and the same argument must apply when *B* is the substrate for transport or when it is the inhibitor of A transport. (3) *A* third amino acid must have the same K_i when used as an inhibitor on either *A* or *B*. The implementation of this test rests upon the success one may have in blocking the various transport systems which have overlapping specificities for the substances under consideration, otherwise, criteria #2 and #3 can never be fulfilled.

Newey and Smyth (1964) found methionine to react exclusively with a discrete system in rat intestine and not with one accepting sarcosine. Proline, on the other hand, was observed to react with both carriers. Hence, when Daniels *et al.* (1969) studied the affinity of proline for the sarcosine carrier, they selectively blocked the methionine pathway with an excess of methionine, in this manner presumably isolating the activity of proline to the sarcosine system. In order to acquire a specific blocking agent among the amino acids, the test substance must be used as an inhibitor of a large variety of amino acid substrates, and the latter in turn must be tested for their inhibitory effects on the transport of the prospective blocking agent. In this manner, Christensen's group (1969a) discovered in BCH a substrate specific for *system L* in Ehrlich cells. Of major importance, BCH allowed them to measure a K_i value for phenylalanine on transport of the former that provides to date the best estimate of the reactivity of phenylalanine with *system L*, such activity presum-

ably being undistorted by affinity of phenylalanine for other transport systems.

Another approach involves the diagnosis of whether the inhibitory action of a given amino acid on the transport of another becomes independent of the inhibitor concentration. Newey and Smyth (1964) found that the inhibition of glycine uptake caused by methionine becomes maximal with equimolar concentrations of these substances, the effect of methionine not increasing up to 60 mM. They reasoned that if methionine inhibits glycine transfer by competition for a common site, then it would appear that this agency becomes saturated with methionine at a concentration of about 15 mM. However, at this concentration of methionine, there is still considerable transfer of glycine, indicating partition of glycine entry into pathways shared and unshared with methionine. Moreover, they have suggested that more than one site is involved in substrate transfer if two saturating inhibitors when present together cause greater inhibition than either amino acid when acting separately at twice the concentration. This additivity principle has been applied by Hillman *et al.* (1968) to distinguish glycine transport systems in renal tubules. When both alanine and proline were added to the incubation medium together, the uptake of glycine was inhibited far more than could be inhibited by twice the concentration of either inhibitor added singly. They concluded that the amino acids appeared to inhibit different glycine agencies.

Another method involves the study of substrate velocity over a wide concentration range (about 15,000 fold) of initial medium concentration (Winter and Christensen, 1965; Hillman *et al.*, 1968). If the substrate is transported by two or more distinct systems with *widely differing* affinities and capacities (K_m and V_{max}, respectively), a two-limbed curve should be revealed when the kinetic data are plotted by the Lineweaver and Burk or velocity *vs.* velocity over concentration methods. A computer program has been developed by Hillman and his colleagues (1968) which permits the mathematical computation of the kinetic constants by summing Michaelis-Menten expressions. They were able to verify or extend this treatment, by analysis of a kinetic situation which yielded a two-limb curve, through the use of competitive inhibitors, singly or in combination. They found that a system shared by alanine and glycine is active primarily at low glycine concentration and that a system shared by proline and glycine has some activity at low concentrations but becomes more important as the glycine concentration is elevated. The unshared system (with either proline or alanine) brings about a significant transport of glycine only at high concentrations, and itself appears to be complex in view of the findings of Scriver and Wilson (1961), who showed additional degrees of inhibition with lysine and with β-alanine. In rat liver slices, the wide concentration range experiment has disclosed

two transport systems for cysteine, as evidenced by a two-limbed Lineweaver-Burk plot (Crawhall and Davis, 1971). One system was observed under conditions of low concentrations of cysteine (0.2 mM or less) and had a high affinity for cysteine (K_m = 0.11 mM) and a low capacity; it was inhibited strongly by glycine and weakly by AIB. The other system operated over a higher concentration range (0.2 mM and above), had a high capacity, but a low affinity for cysteine (K_m of about 4 mM); it was inhibited by glycine, but not by AIB.

George and Baker (1965) suggested that two amino acids showing different patterns of transport along the intestine might be transported by different systems. This approach is based on the idea that different amino acid transport systems might have different longitudinal distributions of activity along the small intestine. These investigators studied the 60 min uptakes of a number of neutral amino acids as a function of position along the rat intestine. The uptake patterns arranged in the order of decreasing similarity followed the sequence: methionine, leucine, alanine, proline, glycine, AIB and betaine. In their view, the large difference between the patterns of betaine and methionine is indicative of the participation of these substrates in two different transport pathways, which are distributed unequally along the intestine. The intermediate shape of the other absorption patterns, they theorize, is expected on the basis of the overlapped transports of the various compounds between the systems functional in the uptake of methionine, on the one hand, and betaine, on the other, as supported by previous data on cross-inhibitions. Furthermore, they showed that in the presence of methionine as a blocking agent, the pattern for proline absorption was shifted toward that for betaine, so that the latter two were barely distinguishable. Thus, proline uptake is probably partitioned between the two pathways. (Baker and George, 1971).

The effect of glucose on amino acid transport under anaerobic conditions can be a useful probe in characterizing transport in a given system (De La Noue, 1970). The absorption of the amino acid is measured under both aerobic and anaerobic conditions to determine the amount of transport which is dependent upon energy derived from aerobic sources. The difference in uptake seen under anaerobic conditions between the glucose-depleted and glucose-enriched systems presumably is an indicator of the energy contribution from glycolysis. De La Noue (1970) found that alanine and methionine in their absorptions show higher uptakes in aerobiosis than in anaerobiosis, even in the presence of glucose. He interpreted these data to mean that glycolysis is not the only energy source for the methionine system; instead, that agency is apparently capable of drawing energy from both aerobic and anaerobic pathways. Such transport is slightly stimulated by glucose in the presence of oxygen,

but strongly in the absence of oxygen. On the other hand, the sarcosin system derives its energy from oxidative metabolism alone; it is stimulate by aerobiosis, but not affected by glucose either under aerobic or ana erobic conditions.

The following is a summary of the criteria which establish the funda mental transport characteristics of a given compound:

1) Determination of velocity as a function of substrate concentra tion over a wide concentration range (15,000 fold).
2) Determination of the progress curve of uptake for measuremen of unidirectional influx and the time of steady state.
3) Determination of the apparent Michaelis constant, K_m an maximum velocity of transport, V_{max}.
4) Determination of the dependence of transport rate on mediun Na^+ concentration.
5) Determination of the distribution ratio for concentrative up take.
6) Determination of the uptake under conditions of anaerobiosis aerobiosis, both with and without glucose as an energy source
7) Determination of inhibitory effects of a wide variety of amino acids and related compounds on substrate uptake.
8) Determination of the inhibitory effects of the test substrate on a wide variety of amino acids.
9) The institution of certain blocking experiments to define more clearly the overlap of substrate transport into the various systems.
10) Application of these criteria to a select number of longitudinal regions of the gut.

SUMMARY

Chicken small intestine appears to possess at least three absorption pathways for neutral amino acids, a transport system for methionine and related aliphatic compounds, a system for glycine, and one for proline and related imino acids. A distinct pathway also exists for cationic amino acids. The characterization of these pathways as well as recent findings on amino acid absorption in mammalian intestine and other tissues has been considered in this report. In general, it can be concluded that avian intestine, with certain exceptions, incorporates the same fundamental pathways of transport that are found in a variety of other tissue systems.

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